POLYMER-LINKER-DRUG CONJUGATES FOR TARGETED DRUG DELIVERY

Related Applications

5   The present application claims priority to provisional application U.S. Serial No.

60/412,760, filed September 23, 2002 which is incorporated herein by reference in its entirety.

Background of the Invention

Despite numerous recent advances in drug delivery technology (Langer, *Nature* 392:5,

10   1998 and Langer, *Science* 293:58, 2001) there remains a need in the art for methods, devices and

compositions for controllably and preferentially delivering drugs to diseased tissues. The ability

to control the precise level and location of drugs in a patient would allow doses to be reduced,

minimize side effects and open new avenues for drug therapy. Targeted drug delivery systems

are particularly desirable in the treatment of tissue specific diseases such as cancer that are

15   currently treated by systemic chemotherapy.

Summary of the Invention

The present invention provides a system for selectively delivering drugs to target tissues.

In preferred embodiments, the target tissues are diseased tissues. The inventive delivery system

20   includes a polymer-linker-drug conjugate. The linker includes a segment that is recognized and

cleaved by a digestive enzyme that is overexpressed in the extracellular space of the target tissue.

The recognition segment is preferably an oligopeptide or oligosaccharide segment. The linker

may also include a spacer that separates the recognition segment from the polymeric carrier

and/or drug. Without wishing to be bound to any particular theory, when the conjugate reaches

the target tissue the recognition segment within the linker is thought to be cleaved by the

5   digestive enzyme. The active drug is thereby released from the conjugate and subsequently

internalized by the cells of the target tissue. The polymeric carrier is preferably hydrophilic,

biodegradable and biocompatible. In preferred embodiments the polymeric carrier is greater in

size than the renal excretion limit. The physiochemical features of the polymeric carrier allow

the conjugate to circulate longer in plasma by decreasing renal excretion and liver clearance.

10   The polymeric carrier may be loaded with any number of drug molecules. In particular it is to be

understood that the conjugate may include a single drug molecule or a plurality of drug

molecules each attached to the polymeric carrier via an inventive linker. It is also to be

understood that any drug molecule whether a small molecule drug or a biomolecular drug (e.g., a

therapeutic protein or nucleic acid) may be delivered using a conjugate prepared according to the

15·   invention.

          The present invention also provides methods of administering the inventive conjugates

and pharmaceutical compositions comprising the inventive conjugates to a patient in need

thereof. The inventive conjugates and/or compositions once prepared can be administered to the

patient by any means known in the art.

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Definitions

"Digestive enzyme": As used herein, a "digestive enzyme" is an enzyme that cleaves

· polymers. Preferably the cleaved polymers are oligopeptides or oligosaccharides. Digestive

enzymes of the present invention exhibit some form of specificity. For example, a digestive

5   enzyme that cleaves oligopeptides will typically exhibit strong selectivity for oligopeptides that

include one or a small subset of amino acid sequences called recognition sequences. As is

further described herein and as is known in the art, the specificity and recognition sequence of a

particular digestive enzyme may be determined by comparing the rate at which it cleaves

different polymers within a given family (e.g., oligopeptides or oligosaccharides having different

10   sequences).

"Oligopeptide": According to the present invention, an "oligopeptide" comprises a

string of at least three amino acid residues linked together by peptide bonds. The terms

"oligopeptide", "peptide", "polypeptide" and "protein" may be used interchangeably. Inventive

oligopeptides preferably contain only natural amino acid residues, although non-natural amino

15   acids (i.e., compounds that do not occur in nature but that can be incorporated into a polypeptide

chain) and/or amino acid analogs as are known in the art may alternatively be employed. Also,

one or more of the amino acid residues in an inventive oligopeptide may be modified, for

example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a

farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation,

20   functionalization, or other modification, etc. In a preferred embodiment, the modifications of the

oligopeptide lead to a more stable oligopeptide (e.g., greater stability to digestion by enzymes in

the gastrointestinal tract). These modifications may include cyclization of the peptide, the

incorporation of D-amino acids, etc. If the modifications are made to an oligopeptide drug

molecule, they should not substantially interfere with the desired biological activity of the

oligopeptide.

5   **"Oligonucleotide"**: According to the present invention, an "oligonucleotide" comprises

a string of at least three nucleotides linked together by phosphodiester bonds. The terms

"oligonucleotide", "polynucleotide" and "nucleic acid" may be used interchangeably. Typically,

an oligonucleotide comprises at least three nucleosides. Oligonucleotides may include natural

nucleosides (i.e., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine,

10   deoxythymidine, deoxyguanosine and deoxycytidine), nucleoside analogs (e.g., 2-

aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, C5-

propynylcytidine, C5-propynyluridine, C5-bromouridine, C5-fluorouridine, C5-iodouridine,

C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine,

O(6)-methylguanine and 2-thiocytidine), chemically modified bases, biologically modified bases

15   (e.g., methylated bases), intercalated bases, modified sugars (e.g., 2′-fluororibose, ribose, 2′-

deoxyribose, arabinose and hexose), or modified phosphate groups (e.g., phosphorothioates and

5′-N-phosphoramidite linkages).

       "Oligosaccharide": The term "oligosaccharide" refers to a polymer of sugars. The

terms "oligosaccharide", "polysaccharide" and "carbohydrate" may be used interchangeably.

20   Typically, an oligosaccharide comprises at least three sugars. The polymer may include natural

sugars (e.g., glucose, fructose, galactose, mannose, arabinose, ribose and xylose) and/or modified

sugars (e.g., 2′-fluororibose, 2′-deoxyribose and hexose).

"Overexpressed": As used herein, a digestive enzyme is "overexpressed" in a tissue

sample if it is present in excess relative to the abundance of that enzyme in at least one other

5   tissue sample (e.g., when comparing a diseased tissue with a healthy tissue from the same

individual). As is well known in the art, there is generally a correlation between overexpression

at the RNA level and overexpression at the protein level. In other words, if a mRNA is

overexpressed then it is highly likely that the corresponding polypeptide is also overexpressed.

Therefore, detection of either mRNA or a corresponding polypeptide is generally sufficient to

10   determine whether a particular polypeptide is overexpressed. However, as is well known in the

art, in certain situations it may be more convenient and/or practical to detect mRNA while in

other situations it may be more convenient and/or practical to detect polypeptides.

Brief Description of the Drawing

15   **Figure 1** is a schematic that illustrates the overall structure of an embodiment of an

inventive conjugate.

Figure 2 is a schematic that illustrates how substrate phage display methodology can be

used to select suitable oligopeptide sequences for use in linkers of inventive conjugates.

Figure 3 is a schematic that illustrates the chemical structure of the free drug

20   doxorubicin.

Figure 4 is a schematic that illustrates the chemical structure of an inventive CM-dextran-oligopeptide-doxorubicin conjugate.

Figures 5A-B are graphs that compare the digestion of four different CM-dextran-oligopeptide-doxorubicin conjugates by pure matrix-metalloproteinase II (MMP-2) at 37 C. The

5   conjugates each include a linker with an oligopeptide in the series IPVGLIG (diamond), IPVGLI (cross), IPVGL (square) and IPVG (triangle). The graphs show the concentration of released peptidyl-doxorubicin as a function of digestion time. The data in Figure 5A were obtained with a doxorubicin-equivalent concentration of 29.5 µM and an MMP-2 concentration of 0.74 µM. The data in Figure 5B were obtained with a doxorubicin-equivalent concentration of 3.2 µM and

10   an MMP-2 concentration of 100 nM.

Figures 6A-F are graphs that compare the cytotoxicity of free doxorubicin (empty squares) and the cleaved peptidyl-doxorubicin (i.e., LIG-doxorubicin, filled squares) on six different human tumor cell lines, namely HT-1080, BT-20, U-87, PC-3, KK-47 and MGH-U1. The graphs show the % survival of tumor cells as a function of the concentration of the

15   doxorubicin-equivalent concentration in the cell culture.

Figure 7 is a schematic that illustrates the chemical structure of an inventive dextran-poly(ethyleneglycol)-oligopeptide-doxorubicin conjugate.

Figure 8 is a schematic that illustrates the chemical structure of an inventive methoxy-poly(ethyleneglycol)-oligopeptide-doxorubicin conjugate

20   **Figure 9** is a schematic that illustrates the chemical structure of the free drug methotrexate.

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Figure 10 is a graph that compares the cytotoxicity of methotrexate-PVG (empty squares), methotrexate-IPVG (empty triangles) and free methotrexate (empty circles) on the human tumor cell line HT-1080. The graphs show the % survival of tumor cells as a function of the concentration of the methotrexate-equivalent concentration in the cell culture.

5   **Figures 11A-C** are graphs that compare the cytotoxicity of free methotrexate (empty circles) and the cleaved peptidyl-methotrexate (i.e., PVG-methotrexate, filled circles) on three different human tumor cell lines, namely HT-1080, BT-20 and RT-112. The graphs show the % . survival of tumor cells as a function of the concentration of the methotrexate-equivalent concentration in the cell culture.

10   **Figures 12A-C** depict a synthetic scheme for preparing inventive dextran-oligopeptide-methotrexate conjugates of the invention.

Figure 13 is a graph comparing the tumor progression after different groups of SCID mice bearing HT-1080 tumors were treated with free methotrexate, a dextran-methotrexate conjugate, a dextran-oligopeptide-methotrexate conjugate, modified dextran or phosphate

15   buffered saline (PBS).

Figure 14 is a graph comparing the body weight change after different groups of SCID mice bearing HT-1080 tumors were treated with free methotrexate, a dextran-methotrexate conjugate, a dextran-oligopeptide-methotrexate conjugate, modified dextran or PBS.

Figure 15 is a graph comparing the tumor progression after two different groups of SCID

20   mice bearing HT-1080 tumors were treated with two dextran-oligopeptide-methotrexate conjugates having different backbone negative charges.

Detailed Description of Certain Preferred Embodiments of the Invention

The present application mentions various patents, published patent applications, scientific articles and other publications. The contents of each such item are hereby incorporated by reference.

5   The present invention provides a system for selectively delivering drugs to target tissues. In preferred embodiments, the target tissues are diseased tissues. The inventive delivery system includes a polymer-linker-drug conjugate. The linker includes a segment that is recognized and cleaved by a digestive enzyme that is overexpressed in the extracellular space of the target tissue. The recognition segment is preferably an oligopeptide or oligosaccharide segment. The linker

10   may also include a spacer that separates the recognition segment from the polymeric carrier and/or drug. Without wishing to be bound to any particular theory, when the conjugate reaches the target tissue the recognition segment within the linker is thought to be cleaved by the digestive enzyme. The active drug is thereby released from the conjugate and subsequently internalized by the cells of the target tissue. The polymeric carrier is preferably hydrophilic,

15   biodegradable and biocompatible. In preferred embodiments the polymeric carrier is greater in size than the renal excretion limit. The physiochemical features of the polymeric carrier allow the conjugate to circulate longer in plasma by decreasing renal excretion and liver clearance. The polymeric carrier may be loaded with any number of drug molecules. In particular it is to be understood that the conjugate may include a single drug molecule or a plurality of drug

20   molecules each attached to the polymeric carrier via an inventive linker. It is also to be understood that any drug molecule whether a small molecule drug or a biomolecular drug (e.g., a

therapeutic protein or nucleic acid) may be delivered using a conjugate prepared according to the invention. The polymeric, linker and drug components of the inventive conjugates are described in greater detail below.

5   **Polymeric carrier**

In certain embodiments, the polymeric carrier allows conjugates and hence drugs to circulate longer in plasma by decreasing renal excretion and liver clearance. Strategies for minimizing body clearance have focused on the liver and kidney because they are known to be the major elimination sites in the body. In this context, Hashida and Takakura have reviewed the

10   current status of macromolecular drug delivery systems (Hashida and Takakura, *Journal of Controlled Release* 31:163, 1994). They related the physiological features of the liver and kidney to the clearance data obtained with macromolecules.

The glomerular capillaries in the kidney are fenestrated, with pores having radii estimated at 20-30 nm. Their basement membranes act as a size and charge barrier which appear to hinder

15   the transport of particles above 6 nm. Consistent with these features, macromolecules with sizes above about 6 nm (MW ~ 50,000 Da) exhibit marked inhibition on renal clearance. Accordingly, in certain embodiments the polymeric carrier may be designed to be greater than this renal excretion limit. It is to be understood however that the inventive conjugates may also include polymer carriers that are smaller than the renal excretion limit.

20   In the liver, the basement membrane is absent and capillaries are characterized by fenestrae of about 100 nm and endothelial gaps from 100-1000 nm. The passage of

macromolecules is therefore relatively unrestricted. However, cellular uptake of these carriers

can be lowered by decreasing interactions between cells and carriers. In general, cationic

molecules tend to bind to cell surfaces and strongly anionic species facilitate receptor mediated

endocytosis. A slightly anionic carrier, for example, carboxymethyl-dextran (CM-dextran),

5　　appears to elicit least uptake by liver cells and its hepatic clearance is similar to that of fluid

phase endocytosis (i.e., ~10 μl/hr in mice).

One additional advantage of using macromolecules as drug carriers in the case of tumor

targeting comes from the abnormality of tumor vasculature (Jain, *Cancer and Metastasis*

Reviews 6:559, 1987). Most capillaries are continuous, with small pore radii of 6.7-8.0 nm and

10　　large pore radii of 20-28 nm. They are found in muscle, lung, skin, subcutaneous tissues, serous

and mucous membranes. Transport of macromolecules above ~ 7 nm across these capillaries is

negligible. In contrast, tumor blood vessels are leaky and the basement membrane is absent. By

observing the tranvascular transport of liposomes under the dorsal skin chamber in tumor-

bearing mice, Yuan et al. determined the cutoff pore size of tumor vessels to be 400-600 nm

15　　(Yuan et al., *Cancer Research* 55:3752, 1995). These features are attributed to the expression of

angiogenic factors and various collagen degrading enzymes (Maeda et al., *Journal of Controlled*

Release 65:271, 2000). Larger drug carriers can therefore selectively reach malignant tissues

while sparing most normal tissues. The liver, bone marrow, spleen and kidney are the key

exceptions.

20　　Maeda and co-workers have further observed that macromolecules are also preferentially

retained in tumor tissues. They conjugated the anti-tumor protein neocarzinostatin to styrene and

maleic acid copolymer (SMANCS) and reported poor recovery of the drug carriers in the effluent

blood and lymph. The explanation for this phenomenon, termed enhanced permeability and

retention (EPR), is not entirely clear. The researchers attribute this effect to the augmented

leakiness of tumor blood vessels and the lack of lymphatic drainage in tumor tissues (Matsumura

5   and Maeda, *Cancer Research* 46:6387, 1986). Essentially, while the molecules can easily

extravasate into the tumor tissue, return to the circulation is blocked.

In addition to controlling the size and charge of drug carrier, Kilbanov and a number of

laboratories have demonstrated the benefit of using poly(ethylene-glycol) (PEG) stealth in

polymeric micelles (Yokoyama et al., *Cancer Research* 51:3229, 1991); nanoparticles (Gref et

10   al., *Science* 263:1600, 1994; Gref et al., *Advanced Drug Delivery Reviews* 16:215, 1995); and

liposomes (Klibanov et al., *FEBS Lett.* 268:235, 1990). A layer of linear hydrophilic polymers

surrounding the carrier in the core provides a shielding effect by avoiding absorption onto the

cell surface in the eliminating organ. With the appropriate molecular weight and density, the

PEG brush also inhibits proteins from interacting with the core through steric hindrance

15   (VertutDoi et al., *Biochimica Biophysica Acta - Biomembranes* 1278:19, 1996; Gref et al.,

Colloids Surfaces B - Biointerfaces 18:301, 2000). These features prolong the circulation of

polymeric carriers in the circulation.

In certain preferred embodiments, the polymeric carriers used in an inventive conjugate

are biocompatible. Biocompatible polymers are not significantly toxic to cells. In order to

20   prevent chromic accumulation of polymeric carriers that are larger than the renal excretion limit

are preferably both biocompatible and biodegradable. Biodegradable polymers are broken down

by the cellular machinery and/or by hydrolysis into components that the cells can either reuse or

dispose of without significant toxic effect. In preferred embodiments, a biodegradable polymer

and its biodegradation byproducts are biocompatible. It is to be understood that any known

biodegradable polymer may be incorporated in a conjugate of the present invention. Preferred

5   polymeric carriers are hydrophilic, e.g., they may include polar groups, such as hydroxyl or

amine groups; anionic groups, such as carboxylate, sulfonate, sulphate, phosphate, or nitrate

groups; or cationic groups, such as protonated amine, quaternary ammonium, or phosphonium

groups. Suitable hydrolytically degradable polymers known in the art include for example,

certain polyesters, polyanhydrides, polyorthoesters, polyphosphazenes and polyphosphoesters.

10   Other biodegradable polymers known in the art, include, for example, certain carbohydrates,

polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, poly(amino acids),

polyacetals, polyethers, biodegradable polycyanoacrylates and biodegradable polyurethanes. For

example, specific biodegradable polymers that may be used in the present invention include but

are not limited to alginate, carboxymethyl-alginate, cellulose, polylysine, poly(lactic acid),

15   poly(glycolic acid), poly(caprolactone), poly(lactide-co-glycolide), poly(lactide-co-caprolactone)

and poly(glycolide-co-caprolactone). Those skilled in the art will recognize that this is an

exemplary, not comprehensive, list of biodegradable polymers. It is further to be understood that

inventive conjugates may comprise block co-polymers, graft co-polymers, or adducts of these

and other polymers.

20   The properties of these and other polymers and methods for preparing them are well

known in the art. See, for example, U.S. Patents Nos. 6,123,727; 5,804,178; 5,770,417;

5,736,372; 5,716,404 to Vacanti; 6,095,148; 5,837,752 to Shastri; 5,902,599 to Anseth;

5,696,175; 5,514,378; 5,512,600 to Mikos; 5,399,665 to Barrera; 5,019,379 to Domb; 5,010,167

to Ron; 4,806,621; 4,638,045 to Kohn; and 4,946,929 to d'Amore; see also Wang et al., *J. Am.

Chem. Soc.* 123:9480, 2001; Lim et al., *J. Am. Chem. Soc.* 123:2460, 2001; Langer, *Acc. Chem.*

5   *Res.* 33:94, 2000; Langer, *J. Control. Release* 62:7, 1999; and Uhrich et al., *Chem. Rev.* 99:3181,

1999. More generally, a variety of methods for synthesizing suitable polymers are described in

Concise Encyclopedia of Polymer Science and Polymeric Amines and Ammonium Salts, Ed. by

Goethals, Pergamon Press, 1980; *Principles of Polymerization* by Odian, John Wiley & Sons,

1991; and *Contemporary Polymer Chemistry* by Allcock et al., Prentice-Hall, 1981.

10

Drug

Any drug whether a small molecule drug or a biomolecular drug (e.g., a therapeutic

protein, nucleic acid or carbohydrate) may be delivered using a conjugate prepared according to

the invention. As used herein, the term "small molecule drug" refers to a molecule, whether

15   naturally-occurring or artificially created (e.g., via chemical synthesis) that has a relatively low

molecular weight and that is not a protein, a nucleic acid, or a carbohydrate. Typically, though

not necessarily, small molecule drugs are monomeric and have a molecular weight of less than

about 1500 g/mol. Preferably, though not necessarily, the drug is one that has already been

deemed safe and effective for use by the appropriate governmental agency or body. For

20   example, drugs for human use listed by the FDA under 21 C.F.R. §§ 330.5, 331 through 361 and

440 through 460; drugs for veterinary use listed by the FDA under 21 C.F.R. §§ 500 through

589, incorporated herein by reference, are all considered acceptable for use in inventive

conjugates.

Classes of small molecule drugs that can be used in the practice of the present invention

include, but are not limited to, anti-AIDS drugs, anti-cancer drugs, antibiotics,

5   immunosuppressants, anti-viral drugs, enzyme inhibitors, neurotoxins, opioids, hypnotics, anti-

inflammatory drugs, anti-histamines, lubricants, tranquilizers, anti-convulsants, muscle relaxants

and anti-Parkinson drugs, anti-spasmodics and muscle contractants including channel blockers,

miotics and anti-cholinergics, anti-glaucoma compounds, anti-parasite and/or anti-protozoal

compounds, modulators of cell-extracellular matrix interactions including cell growth inhibitors

10   and anti-adhesion molecules, vasodilating agents, inhibitors of DNA, RNA or protein synthesis,

anti-hypertensives, analgesics, anti-pyretics, steroidal and non-steroidal anti-inflammatory

agents, anti-angiogenic factors, anti-secretory factors, anticoagulants and/or antithrombotic

agents, local anesthetics, ophthalmics, prostaglandins, anti-depressants, anti-psychotic drugs,

anti-emetics and imaging agents (e.g., for use in computed X-ray tomography, magnetic

15   resonance imaging, positron emission tomography, single photon emission computed

tomography, etc.).

A more complete listing of classes and specific drugs suitable for use in the present

invention may be found in *Pharmaceutical Drugs: Syntheses, Patents, Applications* by Axel

Kleemann and Jurgen Engel, Thieme Medical Publishing, 1999 and the *Merck Index: An*

20   *Encyclopedia of Chemicals, Drugs and Biologicals*, Ed. by Budavari et al., CRC Press, 1996,

both of which are incorporated herein by reference.

As used herein, the term "biomolecular drug" refers to a molecule, whether naturally-occurring or artificially created (e.g., via synthetic or recombinant methods) that has a relatively high molecular weight and that is a protein, a nucleic acid or a carbohydrate. Typically, though not necessarily, biomolecular drugs have a molecular weight of more than about 1500 g/mol.

5   Classes of biomolecular drugs that can be used in the practice of the present invention include, but are not limited to, therapeutic proteins (e.g., enzymes, neurotransmitters, hormones, cytokines, cell response modifiers such as growth factors and chemotactic factors, antibodies, haptens, toxins, interferons, etc.) and therapeutic nucleic acids (e.g., aptamers, ribozymes, anti-sense agents, gene vectors, etc.).

10

Linker

As illustrated in Figure 1, the conjugates include a linker with at least a first and a second end. The linker includes at least one segment that is cleaved when exposed to a digestive enzyme that is expressed in the target tissue. Preferably the digestive enzyme is overexpressed

15   in the target tissue. In preferred embodiments the cleavable segment includes an oligopeptide or an oligosaccharide sequence. The first end of the linker is associated with the polymeric carrier and the second end is associated with the drug molecule. When two entities are "associated with" one another as described herein, they are linked by a covalent or ligand/receptor type interaction. Preferably, the association is covalent. One of ordinary skill in the art will

20   appreciate that a whole host of synthetic methods exist for covalently linking oligopeptide or oligosaccharide segments with the polymeric carriers and drugs of the present invention.

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Without limitation, the Examples presented below describe exemplary synthetic methods in

greater detail. It is also to be understood that any ligand/receptor pair with a sufficient stability

and specificity to operate in the context of the inventive system may be employed to associate

two entities. In particular, the ligand/receptor interaction should be sufficiently stable to prevent

5   premature release of the drug molecule from an inventive conjugate (i.e., prior to enzymatic

cleavage of the linker). To give but an example, a drug (or polymer) may be covalently linked

with biotin and the linker with avidin. The strong binding of biotin to avidin would then allow

for association of the drug (or polymer) and linker. In general possible ligand/receptor pairs

include antibody/antigen, protein/co-factor and enzyme/substrate pairs. Besides biotin/avidin,

10   these include for example, biotin/streptavidin, FK506/FK506-binding protein (FKBP),

rapamycin/FKBP, cyclophilin/cyclosporin and glutathione/glutathione transferase pairs. Other

suitable ligand/receptor pairs would be recognized by those skilled in the art.

The chemical composition of the cleavable segment (e.g., the length and sequence of

amino acids or sugars) will depend for the most part on the motif that is recognized and cleaved

15   by the digestive enzyme in the target tissue. The cleavage motifs are known for a number of

proteases. For example, the table provided in Appendix A lists the cleavage motifs for a range of

secreted or membrane bound proteases that are overexpressed in certain tumor tissues. These are

all potential target enzymes that could be used to trigger release of drug molecules from

inventive conjugates. The Examples describe the optimization of a cleavage segment that is

20   recognized by matrix-metalloproteinase II (MMP-2), a proteinase that is also overexpressed in a

variety of tumors.

A variety of methods are also known in the art that can be used to determine the cleavage

motif of a target enzyme when it is not yet known. These include substrate phage display

libraries (Matthews and Wells, *Science* 260:1113, 1993); positional-scanning peptide libraries

(Rano et al., *Chem. Biol.* 4:149, 1996); and mixture-based peptide libraries (Turk et al., *Nature*

5   *Biotechnology* 19:661, 2001). Positional-scanning synthetic peptide libraries are based on the

detection of cleavage by the release of a C-terminal fluorogenic group. The technique is rapid

and enables analysis of all possible peptide sequences. Currently the libraries only provide

sequence specificity N-terminal to the cleavage site and cannot be used with proteases that

require amino acid residues C-terminal to the cleavage site; this restricts their use largely to

10   serine and cysteine proteases. Mixture-based peptide libraries offer an alternative that can

provide both general applicability and speed. The cleavage site motif C-terminal to the cleavage

site is first determined by partial digestion and N-terminal sequencing of a completely random

peptide mixture. Information from this first round of screening is then used to design a second

library in which strong selected amino acids are fixed, allowing data on sites N-terminal to the

15   cleavage site to be obtained. Reiteration of this process allows an optimal recognition sequence

to be determined. The method has recently been used to determine the cleavage motifs of a

variety of matrix-metalloproteinases (Turk et al., *Nature Biotechnology* 19:661, 2001).

The phage display method has been used to determine peptide substrates for a number of

proteases, for example, plasmin (Hervio et al., *Chemistry & Biology* 7:443, 2000); tissue-type

20   plasminogen activator (Ding et al., *Proc. Natl. Acad. Sci USA* 92: 7627, 1995; Ke et al., *J. Biol.*

Chem. 272:16603, 1997); prostate-specific antigen (Coombs et al., *Chemistry & Biology* 5:475,

1998); and membrane type-1 matrix metalloproteinase (Ohkubo et al., *Biochem. Biophys. Res. Commun.* 266:308, 1999). The methodology for substrate phage display is illustrated in Figure 2. Each phagemid codes for a phage coat protein and a tether with a protease substrate sequence linking the two. To create a phage library, the substrate sequence is randomized by site-directed

5   mutagenesis. Each fusion protein is displayed on the surface of a phage particle. For screening, the phage particles are incubated with the protease of interest. The entire digest is then captured using a support with affinity for the tether. Phage with digestion resistant sequences bind the support via the tether. The tether can be an epitope for monoclonal antibody, a histidine tag or a protein-binding peptide. Phage with labile peptide sequences lack the tether and are not bound

10   by the support. Phage with resistant sequence are subsequently released under elution conditions that disrupt binding between the tether and affinity support. Phage with desired sequences, either labile or resistant to the protease of interest, are propagated in bacteria and the cycle is repeated for enrichment. The incubation conditions can be changed to vary the stringency of the screening criteria. For example, highly labile sequences can be identified by selecting for phage

15   that are released at lower protease concentrations or after shorter incubation times.

For the purposes of the present invention, sequences which are labile to target enzyme but resistant to serum proteins can be preferentially selected from a library of peptides. For example, in a first screen, phage that are released upon incubation with the target enzyme are enriched. These phage are then incubated with serum proteins. Phage which remain on the

20   affinity support are then enriched. The DNA sequence of the phagemids can be determined and translated into an amino-acid sequence.

One potential disadvantage of the phage display screening technique is that the peptides may adopt a different conformation when displayed on the phage surface. The peptides may therefore lose their specificity when used as a linker in the polymer-drug conjugate. Hence it may be necessary to study the kinetics of cleavage of the screened peptides in conjugation with a

5   polymeric carrier of choice, in order to confirm their specificity towards the target enzyme.

For example, in order to evaluate whether a particular linker is suitable for use in an inventive conjugate, a set of polymer-linker-drug conjugates or polymer-linker-dye conjugates may be synthesized for kinetic analysis. Without limitation, one could synthesize mPEG-linker-pNA conjugates (where mPEG is methoxy poly(ethyleneglycol) and pNA is the dye p-

10   nitroanilide). Once the conjugates have been prepared, the kinetics of enzymatic cleavage are readily determined. Preferred conjugates do not release a significant amount of active drug molecules in the blood circulation. The stability of the test conjugates in circulation may be assessed by incubating these with serum (e.g., as described in Trouet, Masquelier et al., *Proc. Natl. Acad. Sci. USA* 79:626-629, 1982). The digestion mixtures are then analyzed by size

15   exclusion HPLC which separates large polymers from small dye or drug molecules. Dye or drug molecules that have been leaked are detected using a spectrophotometer or fluorometer.

The linker sequences are also preferably cleaved at much higher rates by the target enzyme than by non-specific enzymes in normal tissues. Fulfilling this condition is preferred in order to ensure site-specific release of the active drug molecules. To evaluate the specificity of

20   cleavage, the test conjugates are first incubated with the target enzyme(s) and the initial rates of release are measured. A similar experiment is then performed with the non-specific enzymes.

The kinetics can be modeled using the Michaelis-Menten equation, that is,

$$\frac{1}{Vi} = \frac{1}{k_{cat}E_{total}} + (\frac{K_m}{k_{cat}E_{total}})\frac{1}{S_o}$$

where V_i is the initial reaction velocity, S_o is the substrate concentration, presumed to remain relatively constant during the early reaction course, E_{total} is the enzyme concentration, including both bound and non-bound forms, K_m is the Michaelis-Menten constant and k_{cat} is the turnover rate of the substrate-enzyme complex to product. A double reciprocal plot (of $1/V_i$ versus S_o) will yield the kinetic constants. The ratio k_{cat}/K_m is widely used in the literature to characterize the efficiency of enzymatic cleavage. The difference in the k_{cat}/K_m for target enzyme and non-specific enzymes gives a measure of the specificity of the peptide sequence for a particular enzyme. A good candidate for a linker will be cleaved at least 2, 5, 10, or 50 times more efficiently by the target enzyme than by non-specific enzymes (e.g., without limitation serum proteases).

In certain embodiments the linker can also include spacer segments that increase the distance between the bulky polymeric carrier and the drug molecule. For example, a spacer segment can be placed between the cleavage recognition sequence and the polymeric carrier and/or between the cleavage recognition sequence and the drug component. As further explained in the Examples below, an increase in distance between the bulky polymeric carrier and the cleavage recognition sequence may reduce steric hindrance between the digestive enzyme and polymeric carrier and hence facilitate cleavage of the linker. The spacer segment(s) may have any chemical composition. In certain embodiments they can be constructed from a polymer,

e.g., without limitation a synthetic polymer such as poly(ethylene glycol) or a natural

oligosaccharide or oligopeptide (i.e., whereby additional amino acid or sugar residues are added

on either side of the recognition segment). The spacer segments may also result from the

specific covalent or non-covalent means used to associate the polymeric carrier, linker and drug

5   components. For example, the use of a ligand/receptor pair to associate the polymeric carrier

with the recognition segment instead of a direct covalent bond will necessarily increase the

distance between the two components. Preferably a spacer segment is not susceptible to

cleavage by the digestive enzyme of interest. A spacer segment may be modified to increase its

stability to non-specific enzymatic digestion. For example, for an oligopeptide spacer these

10   modifications may include cyclization of the peptide, incorporation of D-amino acids, etc.

It is also worth noting that unless the drug molecule is associated with the linker at the

cleavage site, the cleavage product will necessarily include certain additional residues as

compared to the free drug molecule. Under certain circumstances these additional residues may

interfere with the biological activity of the drug. Accordingly, it may prove advantageous to

15   select an attachment site on the drug molecule for the linker that minimizes any such reduction in

biological activity. These sites are commonly referred to as "bulk tolerant" sites. In addition, it

may prove advantageous to optimize the number of residues that lie between the drug molecule

and the cleavage site of the linker. Note that any reduction in the number or residues may reduce

the specificity of the recognition segment for the digestive enzyme. Accordingly, there may be a

20   need to balance digestion rate, specificity of cleavage and maintenance of biological activity.

Methods and assays for performing suitable optimizations are further described in the Examples.

As mentioned earlier, the linkers of the present invention need not necessarily include a

digestible oligopeptide segment. For example, the linkers may alternatively include digestible

oligosaccharide sequences. Again the chemical composition of the recognition segment (e.g., the

length and sequence of sugar residues) will depend for the most part on the motif that is

5   recognized and cleaved by the digestive enzyme of interest. While the cleavage motifs of

glycosidases have not been characterized in as much detail as those of proteases, a number of

general synthetic routes have been developed in recent years for preparing specific

oligosaccharides. Chemical coupling methods have become increasingly sophisticated to fine-

tune reactivity of reagents by fortuitous choices of anomeric activating group and protecting

10   groups. As a result, oligosaccharide synthesis has become more predictable and reliable and a

number of oligosaccharide libraries have been synthesized in solution and on solid phases

(Flitsch, *Curr. Opin. Chem. Biol.* 4:619, 2000). An exemplary glycosidase in this category is

heparanase. Heparanase is an endo-β-D-glucuronidase that catalyzes the hydrolytic cleavage of

the β-1,4-glycosidic bond between a D-glucuronate and a D-glucosamine in heparan sulfate

15   (Pikas et al., *J. Biol. Chem.* 273:18770, 1998; Jemth et al., *J. Biol. Chem.* 277:30567, 2002).

Interestingly, for the purposes of the present invention, heparanase has been implicated in many

important physiological and pathological processes including tumor cell metastasis, angiogenesis

and leukocyte migration (Vlodavsky et al., *Israel Medical Association Journal*, 2:37, 2000;

Zcharia et al., *Journal of Mammary Gland Biology and Neoplasia*, 6:311, 2001).

20

Targeting agents

In certain embodiments the inventive conjugates may be modified to include targeting agents that will direct an inventive conjugate to a particular cell type, collection of cells, or tissue. Preferably, the targeting agents are associated with the polymeric carrier. A variety of

5   suitable targeting agents are known in the art (Cotten et al., *Methods Enzym.* 217:618, 1993; Torchilin, *Eur. J. Pharm. Sci.* 11:S81, 2000; Garnett, *Adv. Drug Deliv. Rev.* 53:171, 2001). For example, any of a number of different materials which bind to antigens on the surfaces of target cells may be employed. Antibodies to target cell surface antigens will generally exhibit the necessary specificity for the target. In addition to antibodies, suitable immunoreactive fragments

10   may also be employed, such as the Fab, Fab', or F(ab')$_2$ fragments. Many antibody fragments suitable for use in forming the targeting mechanism are already available in the art. Similarly, ligands for any receptors on the surface of the target cells may suitably be employed as targeting agent. These include any small molecule or biomolecule, natural or synthetic, which binds specifically to a cell surface receptor, protein or glycoprotein found at the surface of the desired

15   target cell.

Pharmaceutical compositions

Once the inventive conjugates have been prepared, they may be combined with pharmaceutically acceptable carriers to form a pharmaceutical composition. As would be

20   appreciated by one of skill in this art, the carriers may be chosen based on the route of

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administration as described below, the location of the target issue, the drug being delivered, the

time course of delivery of the drug, etc.

As used herein, the term "pharmaceutically acceptable carrier" means a non-toxic, inert

solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any

5　　type. *Remington's Pharmaceutical Sciences* Ed. by Gennaro, Mack Publishing, Easton, PA,

1995 discloses various carriers used in formulating pharmaceutical compositions and known

techniques for the preparation thereof. Some examples of materials which can serve as

pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose,

glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives

10　　such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered

tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such

as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols

such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as

TWEEN™ 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic

15　　acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; and phosphate buffer

solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and

magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening,

flavoring and perfuming agents, preservatives and antioxidants can also be present in the

composition, according to the judgment of the formulator.

20

Administration

The pharmaceutical compositions of this invention can be administered to a patient by

any means known in the art including oral and parenteral routes. The term "patient", as used

herein, refers to humans as well as non-humans, including, for example, mammals, birds,

5   reptiles, amphibians and fish. Preferably, the non-humans are mammals (e.g., a rodent, a mouse,

a rat, a rabbit, a monkey, a dog, a cat, a primate, or a pig). In certain embodiments parenteral

routes are preferred since they avoid contact with the digestive enzymes that are found in the

alimentary canal. According to such embodiments, inventive compositions may be administered

by injection (e.g., intravenous, subcutaneous or intramuscular, intraperitoneal injection), rectally,

10   vaginally, topically (as by powders, creams, ointments, or drops), or by inhalation (as by sprays).

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions

may be formulated according to the known art using suitable dispersing or wetting agents and

suspending agents. The sterile injectable preparation may also be a sterile injectable solution,

suspension, or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as

15   a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed

are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile,

fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any

bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty

acids such as oleic acid are used in the preparation of injectables. In a particularly preferred

20   embodiment, the inventive conjugate is suspended in a carrier fluid comprising 1% (w/v) sodium

carboxymethyl cellulose and 0.1% (v/v) TWEEN™ 80. The injectable formulations can be

sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating

sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in

sterile water or other sterile injectable medium prior to use.

Compositions for rectal or vaginal administration are preferably suppositories which can

5　be prepared by mixing the inventive conjugate with suitable non-irritating excipients or carriers

such as cocoa butter, polyethylene glycol, or a suppository wax which are solid at ambient

temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and

release the inventive conjugate.

Dosage forms for topical or transdermal administration of an inventive pharmaceutical

10　composition include ointments, pastes, creams, lotions, gels, powders, solutions, sprays,

inhalants, or patches. The inventive conjugate is admixed under sterile conditions with a

pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required.

Ophthalmic formulations, ear drops and eye drops are also contemplated as being within the

scope of this invention. The ointments, pastes, creams and gels may contain, in addition to the

15　inventive conjugates of this invention, excipients such as animal and vegetable fats, oils, waxes,

paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites,

silicic acid, talc and zinc oxide, or mixtures thereof. Transdermal patches have the added

advantage of providing controlled delivery of a compound to the body. Such dosage forms can

be made by dissolving or dispensing the inventive conjugates in a proper medium. Absorption

20　enhancers can also be used to increase the flux of the compound across the skin. The rate can be

controlled by either providing a rate controlling membrane or by dispersing the inventive

conjugates in a polymer matrix or gel.

Powders and sprays can contain, in addition to the inventive conjugates of this invention,

excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and

5   polyamide powder, or mixtures of these drugs. Sprays can additionally contain customary

propellants such as chlorofluorohydrocarbons.

When administered orally, the inventive conjugates are preferably, but not necessarily,

encapsulated. A variety of suitable encapsulation systems are known in the art ("Microcapsules

and Nanoparticles in Medicine and Pharmacy," Edited by Doubrow, M.., CRC Press, Boca

10   Raton, 1992; Mathiowitz and Langer *J. Control. Release* 5:13, 1987; Mathiowitz et al., *Reactive

Polymers* 6:275, 1987; Mathiowitz et al., *J. Appl. Polymer Sci.* 35:755, 1988; Langer *Acc. Chem.

Res.* 33:94, 2000; Langer *J. Control. Release* 62:7, 1999; Uhrich et al., *Chem. Rev.* 99:3181,

1999; Zhou et al., *J. Control. Release* 75:27, 2001; and Hanes et al., *Pharm. Biotechnol.* 6:389,

1995). Preferably the inventive conjugates are encapsulated within biodegradable polymeric

15   microspheres or liposomes. Examples of natural and synthetic polymers useful in the

preparation of biodegradable microspheres include carbohydrates such as alginate, cellulose,

polyhydroxyalkanoates, polyamides, polyphosphazenes, polypropylfumarates, polyethers,

polyacetals, polycyanoacrylates, biodegradable polyurethanes, polycarbonates, polyanhydrides,

polyhydroxyacids, poly(ortho esters) and other biodegradable polyesters. Examples of lipids

20   useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol,

phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides

and gangliosides.

Pharmaceutical compositions for oral administration can be liquid or solid. Liquid

dosage forms suitable for oral administration of inventive compositions include pharmaceutically

5   acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to

an encapsulated or unencapsulated conjugate, the liquid dosage forms may contain inert diluents

commonly used in the art such as, for example, water or other solvents, solubilizing agents and

emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl

alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in

10   particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol,

tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan and mixtures

thereof. Besides inert diluents, the oral compositions can also include adjuvants, wetting agents,

emulsifying and suspending agents, sweetening, flavoring and perfuming agents. As used

herein, the term "adjuvant" refers to any compound which is a nonspecific modulator of the

15   immune response. In certain preferred embodiments, the adjuvant stimulates the immune

response. Any adjuvant may be used in accordance with the present invention. A large number

of adjuvant compounds is known in the art (Allison, *Dev. Biol. Stand.* 92:3, 1998; Unkeless et

al., *Annu. Rev. Immunol.* 6:251, 1998; and Phillips et al., *Vaccine* 10:151, 1992).

Solid dosage forms for oral administration include capsules, tablets, pills, powders and

20   granules. In such solid dosage forms, the encapsulated or unencapsulated conjugate is mixed

with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or

dicalcium phosphate and/or (a) fillers or extenders such as starches, lactose, sucrose, glucose,

mannitol and silicic acid, (b) binders such as, for example, carboxymethylcellulose, alginates,

gelatin, polyvinylpyrrolidinone, sucrose and acacia, (c) humectants such as glycerol, (d)

disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid,

5   certain silicates and sodium carbonate, (e) solution retarding agents such as paraffin, (f)

absorption accelerators such as quaternary ammonium compounds, (g) wetting agents such as,

for example, cetyl alcohol and glycerol monostearate, (h) absorbents such as kaolin and

bentonite clay and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid

polyethylene glycols, sodium lauryl sulfate and mixtures thereof. In the case of capsules, tablets

10   and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and

hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high

molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees,

capsules, pills and granules can be prepared with coatings and shells such as enteric coatings and

15   other coatings well known in the pharmaceutical formulating art.

It will be appreciated that the exact dosage of the inventive conjugate is chosen by the

individual physician in view of the patient to be treated. In general, dosage and administration

are adjusted to provide an effective amount of the inventive conjugate to the patient being

treated. As used herein, the "effective amount" of an inventive conjugate refers to the amount

20   necessary to elicit the desired biological response. As will be appreciated by those of ordinary

skill in this art, the effective amount of inventive conjugate may vary depending on such factors

as the desired biological endpoint, the drug to be delivered, the target tissue, the route of

administration, etc. For example, the effective amount of inventive conjugate containing an anti-

cancer drug might be the amount that results in a reduction in tumor size by a desired amount

over a desired period of time. Additional factors which may be taken into account include the

5   severity of the disease state; age, weight and gender of the patient being treated; diet, time and

frequency of administration; drug combinations; reaction sensitivities; and tolerance/response to

therapy. Long acting pharmaceutical compositions might be administered every 3 to 4 days,

every week, or once every two weeks depending on half-life and clearance rate of the particular

composition.

10   The conjugates of the invention are preferably formulated in dosage unit form for ease of

administration and uniformity of dosage. The expression "dosage unit form" as used herein

refers to a physically discrete unit of conjugate appropriate for the patient to be treated. It will be

understood, however, that the total daily usage of the compositions of the present invention will

be decided by the attending physician within the scope of sound medical judgment. For any

15   conjugate, the therapeutically effective dose can be estimated initially either in cell culture assays

or in animal models, usually mice, rabbits, dogs, or pigs. The animal model is also used to

achieve a desirable concentration range and route of administration. Such information can then

be used to determine useful doses and routes for administration in humans. Therapeutic efficacy

and toxicity of conjugates can be determined by standard pharmaceutical procedures in cell

20   cultures or experimental animals, e.g., ED50 (the dose is therapeutically effective in 50% of the

population) and LD50 (the dose is lethal to 50% of the population). The dose ratio of toxic to

therapeutic effects is the therapeutic index and it can be expressed as the ratio, LD50/ED50.

Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data

obtained from cell culture assays and animal studies is used in formulating a range of dosage for

human use.

5   These and other aspects of the present invention will be further appreciated upon

consideration of the following Examples, which are intended to illustrate certain particular

embodiments of the invention but are not intended to limit its scope, as defined by the Claims.

Examples

10   **Introduction**

The following Examples describe the synthesis and characterization of inventive

conjugates that release anti-cancer drugs in the presence of matrix-metalloproteinase II (MMP-

2), a widely studied proteinase that is overexpressed in a number of cancers (Woessner and

Nagase, *Matrix Metalloproteinases and TIMPS*, Oxford University Press, 2000). The conjugates

15   include linkers with different fractions of an oligopeptide motif that is recognized and cleaved by

MMP-2. The conjugates are characterized by comparing the rate of drug release in the presence

of MMP-2 and fetal bovine serum (an in vitro control for a normal circulation environment).

The cytotoxicity of the conjugates is also compared by exposing various human tumor cell lines

to the cleavage products. In vivo experiments with a mice model are also described.

20   Expression of MMP-2 has been found to be elevated in a number of human epithelial

cancers, including breast (Davies et al., *British Journal of Cancer* 67:1126, 1993); prostate

(Hamdy et al., *British Journal of Cancer* 69:177, 1994); colon (Levy et al., *Cancer Research*

51:439, 1991); ovary (Naylor et al., *International Journal of Cancer* 58:50, 1994); bladder

(Davies et al., *British Journal of Cancer* 67:1126, 1993); and gastric carcinoma (d'Errico et al.,

Mod. Pathol. 4:239, 1991). Elevated expression has been found in both malignant epithelial

5   cells and the surrounding stromal fibroblasts. Moreover, MMP-2 has been implicated with the

angiogenic and metastatic potential of cancer.

Example 1 - *Synthesis of CM-dextran-oligopeptide-doxorubicin conjugates*

Doxorubicin belongs to the class of anthracyclines which kill cells by intercalating within

10   DNA molecules. The structure of doxorubicin is illustrated in Figure 3. Free doxorubicin has a

clinical dose of 60-80 mg/m^2. After intravenous injection, the drug molecules distribute

ubiquitously throughout the body before being quickly eliminated by renal excretion (Cassidy et

al., *Cancer Surveys* 17:315, 1993). Major toxicity is observed within the haemolymphopoietic

system, gastro-intestinal tract, skin, testes and heart. Doxorubicin is used widely in the treatment

15   of cancers, including breast, ovarian, bladder, lung cancers, non-Hodgkin's lymphoma,

Hodgkin's disease and sarcoma.

Carboxymethyl-dextran-oligopeptide-doxorubicin conjugates (see Figure 4), were

synthesized using traditional techniques of peptide coupling and dextran modification.

Oligopeptides of four to seven amino acids were used as linkers between carboxymethyl-dextran

20   (CM-dextran) and doxorubicin. They are listed below in Table 1. The potential cleavage site is

between P1 and P1' (using the nomenclature of Schechter and Berger, *Biochem. Biophys. Res.*

Comm. 27:157, 1967), wherein residues on the N-terminal side of the cleavage site are unprimed

and residues on the C-terminal side of the cleavage site are primed). The four linkers differ

solely in the number of amino acids that are on the C-terminal side of the cleavage site.

Table 1

P4	P3	P2	P1	P1'	P2'	P3'
Ile	Pro	Val	Gly	Leu	Ile	Gly
Ile	Pro	Val	Gly	Leu	Ile	
Ile	Pro	Val	Gly	Leu		
Ile	Pro	Val	Gly			
*Ile	Pro	Val	Ser	Leu	Arg	Ser

*Sequence obtained from Turk et al., *Nature Biotechnology* 19:661, 2001.

Carboxymethyl-dextran was prepared by exposing dextran with an average MW of

40,000 Da to chloracetic acid. Dextran was selected in part because of its biocompatibility

(Sgouras and Duncan, *J. Mater. Sci.: Mat. Med.* 1:61, 1990) and biodegradability (Vercauteren,

Schacht et al., *J. Bioactive and Compatible Polymers* 7:346 1992). In addition, from a synthetic

standpoint, the hydroxyl groups on the dextran backbone provide convenient sites for covalent

association with the oligopeptide recognition segment.

The oligopeptides were synthesized by conventional solid-phase techniques (Bodansky

and Bodansky, *The Practice of Peptide Synthesis*, Springer 1994) and N-terminal protected with

an FMOC group. The N-terminal protected FMOC-peptides (2 eq.) were conjugated with

doxorubicin (1 eq.) in the presence of N,N"-diisopropylethylamine (5 eq.) in DMF using PyBop

(1.9 eq.) and HOBT (2 eq.) as coupling reagents. The reactions were held at room temperature

overnight. From a synthetic standpoint, the aminoribosyl group on doxorubicin allows the drug

molecule to be covalently associated with the oligopeptide linker via an aminolysis reaction.

5   Reversed-phase chromatography employing an acetonitrile gradient in 0.2% trifluoroacetic on a

Vydac C18 column was used to isolate the FMOC-oligopeptide-doxorubicin. Purity as analyzed

by HPLC was greater than 95%. Deprotection of FMOC was completed in 5 minutes using 10%

piperidine in DMF followed by quenching on ice with a mixture of trifluoroacetic

acid/pyridine/DMF (3:7:20). After deprotection, oligopeptide-doxorubicin was covalently linked

10   to pre-activated dextran carrier in the presence of N-ethoxycarbonyl-2-ethoxy-1,2-

dihydroquinoline (EEDQ).

Size exclusion chromatography on a Sephadex G25 column was then used to separate

carboxymethyl-dextran-oligopeptide-doxorubicin conjugates from unreacted oligopeptide-

doxorubicin complexes. Conjugate purity as analyzed by HPLC was greater than 99%. The

15   loading density of the conjugate was about 1%.

The oligopeptide sequences in Table 1 were based on the MMP-2 cleavage motifs that

have been determined using mixture-based oriented peptide libraries (Turk et al., *Nature*

Biotechnology 19:661, 2001). The optimal MMP-2 cleavage motif determined by Turk et al.,

was Ile-Pro-Val-Ser-Leu-Arg-Ser (P4 through P3', shown on the bottom of Table 1). However,

20   the presence of reactive side chains on serine and arginine (i.e., at positions P1, P2' and P3',

shown in bold in Table 1) and the fact that doxorubicin is labile to harsh deprotection

procedures, posed problems for synthesis. As a result, the second best amino acids at positions

P1, P2' and P3' with non-reactive side chains were used in synthesizing the inventive

carboxymethyl-dextran-oligopeptide-doxorubicin conjugates (i.e., an Ile-Pro-Val-Gly-Leu-Ile-

Gly motif was selected instead).

5

Example 2 - *Peptidyl doxorubicin release in the presence of MMP-2*

Two opposing factors determine the optimization of the length of an oligopeptide

recognition segment, namely the cytotoxicity of the released drug molecule and the sensitivity of

the linker to cleavage by the digestive enzyme. If doxorubicin is linked to the oligopeptide

10   adjacent to the cleavage site (i.e., at the P1' position), the released molecule is free doxorubicin –

a fully active drug. The cytotoxicity may probably be attenuated if doxorubicin is linked further

away from the cleavage site (e.g., at the P2' or P3' positions). However, past studies on matrix-

metalloproteinase specificity have implied that substrate sites from P3 to P3' are all required for
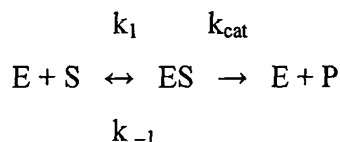
cleavage to occur.

15   Enzymatic release of doxorubicin from the conjugates prepared in Example 1 was

therefore measured. The conjugates were incubated with purified active MMP-2 and the results

are illustrated in Figure 5A (high enzyme and substrate concentrations) and Figure 5B (low

enzyme and substrate concentrations). Under both sets of conditions, the rate of doxorubicin

release followed the trend IPVGLIG >> IPVGLI > IPVGL > IPVG, i.e., in agreement with the

20   literature suggesting that substrate positions from P3 to P3' are important for MMP recognition

and cleavage. Conjugates with the shortest oligopeptide (IPVG) were insensitive to the presence

of MMP-2 even under conditions with high enzyme and high substrate concentrations. In a

control set-up wherein PBS was added instead of MMP-2, no detectable release was observed for

all conjugates confirming that release was triggered by MMP-2.

In order to further characterize the enzymatic cleavage of the linker IPVGLIG, the rate

5   constant for cleavage was also determined according to the Michaelis-Menten model, i.e., based

on the equilibrium model:

$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \overset{k_{cat}}{\longrightarrow} E + P$$

10   where $K_m = k_{-1}/k_1$ is termed the Michaelis-Menten constant, k_{cat} is the turnover rate constant and

k_{cat}/K_m is termed the specificity constant and provides a measure of the rate of enzymatic

cleavage.

As shown in Table 2, despite the attachment of doxorubicin the rate of enzymatic

cleavage for IPVGLIG-doxorubicin compares favorably with that of the optimal cleavage motif

15   IPVSLRSG (Turk et al., *Nature Biotechnology* 19:661, 2001). However, when IPVGLIG-

doxorubicin was covalently attached to carboxymethyl-dextran, the specificity constant

decreased by two orders of magnitude. This suggested that a strong steric hindrance effect was

being imposed by the polymer backbone.

20

Table 2

Species	Structure	k_{cat}/K_m ($M^{-1}.s^{-1}$)
Optimal Linker*	IPVSLRSG	$0.82 \pm 0.06 \times 10^5$
Linker-Drug	IPVGLIG-doxorubicin	1.40×10^5
Polymer-Linker-Drug	CM-dextran-IPVGLIG-doxorubicin	1.77×10^3

*Value obtained from Turk et al., *Nature Biotechnology* 19:661, 2001.

As shown in Table 3, when ionic strength was increased under digestion conditions, the specificity constant increased. This trend suggested that at least part of the hindrance was due to

5   electrostatic repulsion between the negatively-charged polymer backbone and the active site of MMP-2. These results indicate that the sensitivity of the polymer-linker-drug conjugate towards MMP-2 should be improved by decreasing the charge density of the polymer-backbone. This is further demonstrated in Example 8.

Table 3

Salt concentration (NaCl, M)	k_{cat}/K_m ($M^{-1}.s^{-1}$)
0.01	7.50×10^1
0.10	1.15×10^2
0.20	1.09×10^3
0.50	4.47×10^3

Example 3 - *Stability of CM-dextran-oligopeptide-doxorubicin conjugates in serum*

To achieve efficient targeting, it is preferable that premature release of active drug

molecules in the blood stream is minimal. To this end, the CM-dextran-oligopeptide-

doxorubicin conjugates of Example 1 were incubated at 37 C with fetal bovine serum for up to

5   24 hours. All four conjugates were relatively stable in the serum, exhibiting less than 5% release

of doxorubicin.

Example 4 - *In vitro cytotoxicity of peptidyl doxorubicin*

The cytotoxicity of the cleavage product from CM-dextran-IPVGLIG-doxorubicin (i.e.,

10   the conjugate most sensitive to cleavage by MMP-2 in this series) was measured. Confirmed by

mass spectroscopy to be LIG-doxorubicin, the cleavage product was cytotoxic to a number of

human tumor cell lines, namely HT-1080 (a human fibrosarcoma line, Figure 6A), BT-20 (a

human breast carcinoma line, Figure 6B), U-87 (a human glioblastoma cell line, Figure 6C), PC-

3 (a human prostate tumor cell line, Figure 6D), KK-47 (a human bladder tumor cell line, Figure

15   6E) and MGH-U1 (a human bladder cell line, Figure 6F). Open circles in Figures 6A-F

represent free doxorubicin and closed circles represent LIG-doxorubicin.

Cell survival was assayed using an indirect colorimetric immunoassay (MTT assay, e.g.,

see Alley et al., *Cancer Res.* 48:589, 1988). The tetrazolium salt MTT is metabolized by NAD-

dependent dehydrogenase to form a colored reaction product and the amount of dye formed

20   directly correlates with the number of viable cells. The formula for calculating % survival is

given by:

% survival = $(T_i - T_z)/(C - T_z)$ if $T_i \geq T_z$ or

% survival = $(T_i - T_z)/T_z$ if $T_i < T_z$

where T_i = Net MTT reading of test growth in the presence of drugs; T_z = Net MTT

reading at time zero with respect to the drug test; and C = Net MTT reading of control growth

5   (see Grever et al., *Seminars in Oncology*, 19:622, 1992 and Boyd and Paull, *Drug Development*

Research 34:91, 1995).

Overall, LIG-doxorubicin was less potent than the free drug doxorubicin. Doxorubicin

exerts its cytotoxicity by intercalating DNA and the ribosyl amino group is important in

stabilizing DNA binding through hydrogen bonding. Modification of this amino group may

10   therefore explain the reduction in activity.

Example 5 - *Synthesis of a dextran-poly(ethyleneglycol)-oligopeptide-doxorubicin conjugate*

The following describes the synthesis of a slightly different conjugate that also includes

the drug doxorubicin. The polymeric backbone is dextran grafted with poly(ethyleneglycol)

15   (PEG). Doxorubicin is linked to PEG via a cleavable oligopeptide sequence. Like dextran, PEG

is biocompatible (Sgouras and Duncan, *J. Mater. Sci.: Mat. Med.* 1:61, 1990). However, unlike

dextran it is not biodegradable and hence the use of this polymer is limited to a size below the

renal excretion limit (Yamaoka et al., *J. Pharm. Sci.* 83:601, 1994). Pegylation is known to

prolong circulation time in the blood stream, reduce immunogenicity and increase solubility.

20   These advantages are highly dependent on the hydrophilicity imparted by the polymer. With

these benefits, PEG-L-asparaginase has been approved for clinical use in the United States to

treat leukemia (Keating et al., *Leukemia Lymphoma* 10:153, 1993).

The oligopeptide linkers are synthesized by conventional solid-phase techniques

(Bodansky and Bodansky, *The Practice of Peptide Synthesis*, Springer 1994) and the N-terminus

5   is protected by FMOC. The ribosyl amino group of doxorubicin is condensed with the carboxyl

terminus of the oligopeptide in the presence of PyBop and HOBT in DMF. The FMOC

protection group on the oligopeptide is then removed using 10% piperidine to expose the free N-

terminus, which is then attached to a heterogeneously substituted bivalent PEG-FMOC-NH-

PEG-NHS- using EDC as the conjugating reagent in aqueous medium. After conjugation, the

10   FMOC group on PEG is removed using 10% piperidine to afford the resulting intermediate NH_2-

PEG-oligopeptide-doxorubicin. In the presence of EDC in aqueous phase, this intermediate is

covalently linked via amide linkage to carboxymethyl dextran (prepared as in Example 1)

forming a CM-dextran-(PEG-oligopeptide-doxorubicin)$_n$ complex. Subsequently, the remaining

carboxymethyl groups on dextran are reacted with excessive amount of mPEG-NH_2 using EDC

15   as conjugating reagent. The chemical structure of the conjugate is illustrated in Figure 7.

As noted above, the ratio of PEG to PEG-oligopeptide-doxorubicin on the dextran core

may affect the relative rates at which the final conjugate is engulfed by cells or cleaved by

digestive enzymes in the extracellular space. Indeed, the surface hydrophobicity of the conjugate

may have a bearing on the endocytosis rate of the conjugate. Hydrophobic particles are

20   generally engulfed more quickly than hydrophilic particles. For this reason, micro- and

nanoparticles in drug delivery applications are frequently coated with hydrophilic PEG to

elongate their circulation by evading the engulfment by the retinoendothelial system (RES).

With dextran and PEG as the polymer backbone, the conjugates will have a hydrophilic

nature. However, loading of hydrophobic drugs could alter the surface characteristics of the

conjugates and increase the clearance by the RES (Kataoka, p. 49 in *"Targetable polymeric*

5   *drugs. Controlled drug delivery: challenges and strategies"* Ed. by Park, American Chemistry

Society, 1997). The hydrophobicity of conjugates can be compared by measuring the contact

angle with water on a surface coated with the conjugates. The endocytotic rate of conjugates can

also be measured by placing radioactively labeled conjugates in a Kupffer cell culture. Kupffer

cells are chosen since they are the major phagocytes in the RES. At specified time points, cell

10   samples are removed, washed extensively and homogenized. The amount of conjugate engulfed

is then proportional to the radioactivity detected by a scintillation counter.

Example 6 - *Synthesis and characterization of a methoxy-poly(ethyleneglycol)-oligopeptide-*
doxorubicin conjugate

15   An IPVGLIG-doxorubicin complex was prepared following the procedures described in

Example 1 and then covalently attached to a linear flexible polymer, namely methoxy-

poly(ethyleneglycol)-NHS ester (mPEG-NHS, nominal MW of ~ 20,000 Da from Shearwaters

Polymer) in the presence of N,N''-diisopropylethylamine in acetonitrile. The reaction was held

overnight at room temperature. The conjugate methoxy-poly(ethyleneglycol)-oligopeptide-

20   doxorubicin was purified by reversed phase chromatography over an acetonitrile gradient in

0.2% trifluoroacetic acid using a Vydac C18 column. A schematic representation of this

conjugate is shown in Figure 8. The digestion kinetics of mPEG-IPVGLIG-doxorubicin

conjugate by MMP-2 was measured and found to be equivalent to that of IPVGLIG-doxorubicin

complex, thus much faster than that of CM-dextran-IPVGLIG-doxorubicin conjugate (see

Example 2).

5   The three-dimensional structure of the catalytic domain of MMP-2 was examined

(available online in the Brookhaven protein bank). The substrate binding site consists of a

groove that is enclosed in the enzyme interior and has space for about six amino acids. Although

the two ends of the groove are exposed, directly tethering the recognition segment to a large

polymer backbone is likely to give rise to significant steric hindrance. Comparing the results of

10 CM-dextran-IPVGLIG-doxorubicin and mPEG-IPVGLIG-doxorubicin suggests that

incorporation of a flexible spacer (e.g., polyethylene oxide) between the digestible oligopeptide

and the polymer backbone (e.g., dextran or CM-dextran) may improve release of drugs from

such conjugates. Alternatively, these results suggest that one could construct a conjugate using a

PEG star polymer with oligopeptide-drugs linked to the outer ends for better presentation of the

15 digestible oligopeptide.

Example 7 - *Methotrexate as an alternative to doxorubicin*

   As described earlier, in certain preferred embodiments the activity of a drug that is to be

incorporated into a conjugate of the present invention is minimally affected by the covalent

20 attachment of several amino acids (i.e., the peptidyl remnants of linker cleavage). In this

context, Rosowsky has reported that methotrexate (an anti-metabolite that is used to treat cancer

and arthritis) bears "bulk tolerance" at the γ-carboxyl of its glutamic acid (Rosowsky et al., *J. Med. Chem.* 24:1450, 1981). Methotrexate is effective against a number of human tumors, for example, lymphoblastic leukemia in children, chriocarcinoma and related trophoblastic tumors in women, osteosacroma and carcinomas of breast, head, neck, ovary and bladder. The major side

5   effect is toxicity in gastrointestinal tract and bone marrow (Chamber et al., p. 1389 in *"Goodman and Gilman's: The Pharmacological Basis of Therapeutics"*, Ed. by Hardman et al., McGraw-Hill, 2001). The structure of methotrexate is illustrated in Figure 9.

The cytotoxicity of methotrexate and various peptidyl-methotrexates were compared in order to assess bulk tolerance. The peptidyl-methotrexates were prepared by linking short

10   peptides of three to four amino acids to methotrexate. The peptides were chosen to represent appropriate cleavage fragments from the MMP-2 sensitive substrate peptide IPVGLIG that was characterized in Examples 1-2. Methotrexate was covalently coupled to the N-terminal end of the peptides via the γ-carboxyl of its glutamic acid (Nagy et al., *Proc. Nat. Acad. Sci. USA* 90:6373, 1993). The cytotoxicity results with HT-1080 tumor cells (a human fibrosarcoma line)

15   are presented in Figure 10 as a plot of % cell survival against equivalent drug concentration. Cytotoxicity was assayed as described above in Example 4. The cytotoxicity of methotrexate was still reduced when attached to the short peptides, but to a lesser degree that with doxorubicin (see Figures 6A-F). As shown in Figures 11A-C, methotrexate-PVG was found to be cytotoxic when tested on three human tumor cell lines, namely HT-1080 (a human fibrosarcoma line,

20   Figure 11A), BT-20 (a human breast carcinoma line, Figure 11B) and RT-112 (human urinary

bladder transitional carcinoma line, Figure 11C), albeit with a certain degree of neutralization as compared to free methotrexate. Example 8 describes the synthesis of certain dextran-oligopeptide-methotrexate conjugates.

5   **Example 8** - *Synthesis of dextran-oligopeptide-methotrexate conjugates*

Dextran-oligopeptide-methotrexate conjugates were synthesized following the route of Figures 12A-C. The scale of synthesis and the average step yield are also shown. The synthesis route may be modified to prepare conjugates with different oligopeptide recognition segments and different backbone charges. By tuning these parameters, the new conjugate may be

10   optimized in terms of sensitivity against a digestive enzyme of interest and stability in circulation.

1. Materials and Methods

a. Materials

15   Dextran T-70 (nominal MW of 70,000 Da) was obtained from Pharmacia. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and ethanolamine were obtained from Alfa Aesar. O-Bis-(aminoethyl)ethylene glycol trityl resin, PyBop and HOBT were obtained from Nova Biochem. Glutamic acid-α-OtBu was purchased from Bachem. Trifluoroacetic acid (TFA), dimethylformamide (DMF), dimethylsulfoxide (DMSO) and acetonitrile (ACN) were

20   obtained from EM Science. Chloroacetic acid, N,N'-diisopropyl(ethylamine) (DIPEA), BOP, 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) and other chemicals were from Sigma.

Purified active human MMP-2 was purchased from Calbiochem. Precast protein gels

with 10% gelatin were obtained from Biorad. All cell culture media and reagents were ordered

from Gibco. Human tumor cell lines HT-1080, U-87 and BT-20 were obtained from American

Type Cell Culture. Human bladder tumor cell line RT-112 was a gift from the laboratory of

5 Professor Marsha Moses of the Children's Hospital (Boston, MA).

b. Reversed phase analytical assay for monitoring process yield and product purity

A Vydac silica C18 0.21 mm ID x 25 cm L column was connected to an Agilent 1100

series HPLC solvent delivery system equipped with a UV detector. The method ran at 0.5

10 ml/min with mobile phase A as 0.2% TFA in water and mobile phase B as 0.2% TFA in

acetonitrile. A sample of 10 µL was injected onto a pre-equilibrated column followed by 5

minutes of wash with A. A gradient ramped from 0-100% B in 20 minutes. Detection for

methotrexate-containing moieties was made at A307. A calibration curve was constructed

correlating the peak areas in chromatograms and concentrations of methotrexate standard.

15

c. Two column size-exclusion assay for enzyme kinetics study

A Tosohaas PW3000 7.8 mm ID x 30 cm L column followed by a PW2000 7.5 mm ID x

30 cm L column were connected to an Agilent 1100 series HPLC solvent delivery system

equipped with a UV detector. Isocratic elution was performed with 20% acetonitrile and 3.6 X

20 phosphate buffered saline in water at 0.8 ml/min. Detection for methotrexate-containing

moieties was made at A307. The default sample injection volume was set at 10 µL. A

calibration curve was constructed correlating the peak areas in chromatograms and

concentrations of methotrexate standard.

d. One column size-exclusion assay for monitoring process yield and product purity

5   A Tosohaas PW3000 7.8 mm ID x 30 cm L column was connected to an Agilent 1100

series HPLC solvent delivery system equipped with a UV detector. Isocratic elution was

performed with 20% acetonitrile and 3.6 X phosphate buffered saline in water at 0.8 ml/min.

Detection for methotrexate-containing moieties was made at A307. The default sample injection

volume was set at 100 μL. A calibration curve was constructed correlating the peak areas in

10   chromatograms and concentrations of methotrexate standard.

e. Zeta potential measurement

Dextran, dextran-methotrexate conjugates and dextran-oligopeptide-methotrexate

conjugates were dissolved in DI water at 10 mg/ml. Measurement was performed with a Zeta-

15   analyzer from Brookhaven Instruments Corp.

f. Measurement of kinetic parameters for MMP-2 digestion of conjugates

Conjugates of interest were dissolved in assay buffer (10 mM $CaCl_2$, 0.2 M NaCl in 50

mM Tris pH 7.5) at a range of concentrations (5, 10, 20, 50 and 100 μM). For each substrate

20   concentration, active MMP-2 at a final concentration of 9.7 nM was added and the mixture was

incubated at 37 C. At specific time points, the reaction mixture was quenched with EDTA

solution to a final concentration of 20 mM. The mixture was assayed by two-column size

exclusion HPLC and the cleavage product peak was quantified to determine the reaction rate.

The initial rate was based on the time taken to cleave the first 20% of the conjugate. Triplicate

measurements were made at each time point for each substrate concentration. Linear regression

5   was performed on a double reciprocal plot of $1/V_i$ versus $1/S_o$ to determine the specificity

constant k_{cat}/K_m according to the Michaelis-Menten equation.

2. *Synthesis*

        a. *Preparation of carboxymethyl dextran (CM-dextran) [structure iv, Figure 12]*

10   The procedure used was based on the method by Nogusa for activating pullulan (Nogusa

et al., *Chemical & Pharmaceutical Bulletin* 43:1931, 1995). 10 g of dextran T-70 was dissolved

in 100 ml of 6 N sodium hydroxide solution. 30.2 g of chloroacetic acid was added to the

solution and held at 70 C for 2-3 hours. Elevated temperature was necessary for this reaction.

The reaction mixture was removed from heat and checked for pH. Acetic acid was used to

15   neutralize the pH if necessary. The solution was then dialyzed against DI water using a

Spectrapor regenerated cellulose membrane with a molecular weight cutoff at 3000-8000 for 2

days at 4 C and lyophilized for storage. The degree of carboxymethyl substitution was

determined by analysis of sodium content. Atomic absorption analysis was performed at

Quantitative Technologies Inc. (Whitehouse, NJ). The degree of substitution was consistently at

20   50% (+/- 2%).

b. Synthesis and purification of methotrexate-alpha-OtBu [structure i, Figure 12]

MTX-α-(OtBu) was prepared from 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) and

glutamic acid-α-OtBu using BOP as the conjugating reagent, following the procedure of Nagy

(Nagy et al., *Proc. Natl. Acad. Sci. USA* 90:6373, 1993). Crude MTX-α-(OtBu) was precipitated

5   by centrifugation in a 1:1 mixture of cold ether/ethyl acetate. The material was further purified

by HPLC on a Vydac C18 preparative scale column using a gradient from 0-100% acetonitrile

with 0.2% TFA. The organic solvent in product fractions was removed with RotaVap and the

purified material was subsequently lyophilized. The product purity was confirmed to be ≥ 95%

and the amount was quantified by analytical scale reversed-phase HPLC. The average retention

10   factor was 6.14.

c. Synthesis of jeffamine-oligopeptide-methotrexate(OtBu) [structure iii, Figure 12]

Standard FMOC peptide synthesis in solid phase was carried out on O-Bis-

(aminoethyl)ethylene glycol trityl resin by the MIT Biopolymer Lab (Cambridge, MA). As in

15   conventional peptide synthesis, the peptide chain grew from carboxyl to amino terminals. After

the last amino acid was loaded to the chain, FMOC was removed and the oligopeptide with a free

amino terminal was exposed. The carboxyl terminus remained attached to the solid resin via a 6

carbon polyethylene oxide spacer.

To couple MTX-α-OtBu to the amino terminal of the oligopeptide chain, MTX-α-OtBu

20   (2.5 eq.) was dissolved in DMF with DIPEA (2.5 eq.). PyBop (2.5 eq.), HOBT (2.5 eq.) and

another 2.5 eq. of DIPEA were added to activate the γ-carboxyl of MTX-α-OtBu for about 15

minutes. More DMF was added such that the final concentration of MTX-α-OtBu was about 0.1

M. The mixture was added to the resins loaded with oligopeptide and stirred at room

temperature overnight. The reaction was confirmed for completeness the next day using the

ninhydrin test. Excessive reactants were washed away from resins with DMF, DCM and

5   methanol. Resins were dried thoroughly.

        Jeffamine-oligopeptide-MTX(OtBu) was cleaved from the trityl resin by suspending the

resins in 2% TFA in DCM for 10 minutes. This step was repeated 3 times. To collect all the

cleaved product, the resins were further washed with DCM and methanol and the step was

repeated until no further yellow color was observed (the yellow color is indicative of the

10   presence of the MTX(OtBu) containing product). Organic solvent was then removed from the

collected product by RotaVap. The crude material was resuspended in water and purified by

HPLC over a 0-100% acetonitrile gradient with 0.2% TFA using a 95 ml Vydac C18 preparative

column. Product fractions were lyophilized after organic solvent was removed by RotaVap.

Product purity was found to be $\geq$ 95% and the amount was quantified by analytical scale

15   reversed phase HPLC. The average retention factor was 6.71. Mass spectroscopy confirmed the

identity of product (with Pro-Val-Gly-Leu-Ile-Gly as oligopeptide in jeffamine-oligopeptide-

MTX(OtBu), calculated mass: 1177.3; observed mass: 1177.7).

        d. Synthesis of jeffamine-methotrexate(OtBu) [structure iii', not shown]

20   The procedure was similar to that described above in (c) except that no oligopeptide was

loaded onto the resin. MTX-α-OtBu was directly coupled to O-Bis-(aminoethyl)ethylene glycol

trityl resin using the same protocol. The product was found to be ≥ 95% by reversed-phase

HPLC. The average retention factor was 5.81. Mass spectroscopy confirmed product identity

(calculated mass: 640.6; measured mass: 640.6).

5   *e. Synthesis and purification of dextran-oligopeptide-MTX [structure vii, Figure 12]*

To couple jeffamine-oligopeptide-MTX(OtBu) to CM-dextran, 0.5 mmole of jeffamine-

oligopeptide-MTX(OtBu) [structure iii] was dissolved in 1.9 ml DI water and then added to 4 g

of CM-dextran [structure iv] dissolved in 6.7 ml of DI water. 4.7 g of EDC was added to the

mixture with stirring. Reaction was held overnight at room temperature. Another 4.7 g of EDC

10 was added the next day and the reaction continued overnight to form CM-dextran-oligopeptide-

MTX(OtBu).

To block unreacted carboxymethyl groups on the dextran carrier, 30 ml of ethanolamine

was first neutralized to pH 6-7 using 5 N HCL in dibasic phosphate buffer on ice. The mixture

was added to the CM-dextran-oligopeptide-MTX(OtBu) followed by 47 g of EDC. The reaction

15 mixture was stirred at room temperature overnight. Dextran-oligopeptide-MTX(OtBu) after

neutralization was precipitated by centrifugation in methanol.

To remove the protection group OtBu, modified dextran-oligopeptide-MTX(OtBu) was

resuspended in 40 ml strong trifluroacetic acid by vigorous stirring and held at room temperature

for 3 hours. The deprotection step was quenched on ice by diluting in 0.1 M phosphate buffer

20 and neutralizing the pH with 5 N sodium hydroxide. Extensive diafiltration was performed using

Millipore Pellicon XL diafiltration cassettes of regenerated cellulose with 10,000 MWCO to

remove excessive reactants and byproducts of reaction. After purification, the product in the

retentate was concentrated and lyophilized for storage. The product purity was confirmed to be

$\geq$ 99% and the loading density was quantified by size exclusion HPLC. The loading density was

estimated to be at 1 +/- 0.2% (mole % per unit glucose unit).

5

f. Synthesis and purification of dextran-MTX [Structure vii', not shown]

The procedure was similar to that described above in (e) except that jeffamine-

MTX(OtBu) was used instead of jeffamine-oligopeptide-MTX(OtBu).

10　*3. Discussion*

a. Synthesis of oligopeptide-drug product

As described in Example 7, methotrexate was selected to form the drug component of

these inventive conjugates because it exhibits bulk tolerance at the γ-carboxyl group. The less

tolerant α-carboxyl group was protected throughout the synthesis process by preparing MTX-α-

15　(OtBu) [structure i] from APA. The α-protected methotrexate was conjugated to the recognition

oligopeptide in a solid phase reaction. Trityl resin was chosen as the solid phase support so that

the oligopeptide-drug product [structure iii] could be released from the resin using dilute acid

that did not affect the tert-butyl protecting group on methotrexate. The tert-butyl group was

removed in the last reaction step using strong TFA cleavage (step 3A, Figure 12).

20

     b.　Addition of a spacer decreases steric hindrance between the recognition segment and

polymeric carrier

     Experience with the conjugates of Example 1 indicated that lack of spacing between the

oligopeptide recognition sequence and the polymer carrier may reduce the rate of enzymatic

5   cleavage as a consequence of steric hindrance.　The recognition segment and polymeric carrier of

the methotrexate conjugates were therefore separated by a ten atom hydrophilic and flexible

spacer called Jeffamine (2,2'-(ethylenedioxy)diethylamine).　A special trityl resin, O-Bis-

(aminoethyl)ethylene glycol trityl resin [structure ii] was used as the solid support thereby

enabling the recognition segment to be directly attached to Jeffamine on the solid phase, thus

10   simplifying the purification task.

     Jeffamine also aided in coupling the polymeric carrier [structure iv] and the oligopeptide-

drug product [structure iii].　Indeed, the selected oligopeptide sequence for MMP-2 recognition

includes hydrophobic amino acids (Pro-Val-Gly-Leu-Ile-Gly) and the protected drug MTX-α-

(OtBu) has limited water solubility.　On the other hand, the polymeric carrier is composed of

15   hydrophilic CM-dextran.　Attempts to couple the oligopeptide-drug and polymeric carrier in the

presence of EDC, revealed that the concentration of amine components [structure iii] and

carboxyl components [structure iv] affected conjugation yield.　Table 4 lists the conjugation

yields that were obtained when the amine and/or carboxyl concentrations were varied (all other

reaction conditions were unchanged).

20

Table 4

Amine concentration (M)	Carboxyl concentration (M)	Conjugation Yield (%)
0.02	0.66	76
0.02	0.18	41
0.02	0.09	29
0.02	0.02	15
0.01	0.66	25

These observations are thought to be caused by the relatively short half-life of the EDC-

5 activated carboxyl. Indeed, when EDC is unable to encounter an amine component for reaction,

the active ester becomes inactivated (Hermanson, *Bioconjugate Techniques*, Academic Press,

1996). The hydrophilic character of the jeffamine spacer may increase the water solubility of the

oligopeptide-drug product [structure iii] thereby enabling conjugation in aqueous phase to

achieve reasonable yield (step 2A, Figure 12).

10

c. Varying the backbone negative charge of the polymeric carrier

Experience with the conjugates of Example 1 indicated that the rate of MMP-2 cleavage

tends to increase as the ionic strength of the digestion medium is increased (see Table 3,

Example 2). Debye-Hückel theory would suggest that this is caused by electrostatic repulsion

15 between the enzyme and conjugate because of the negative charge on the CM-dextran backbone

(Chau and Langer, *Second International Symposium on Tumor Targeted Delivery Systems* in

Rockville, MD, "Important factors in designing targeted delivery of cancer therapeutics via

MMP-2 mediation", 2002). Unreacted carboxymethyl groups were therefore blocked with

excess ethanolamine [structure v], a neutral and hydrophilic molecule (step 2B, Figure 12). The

5   charge on the polymer backbone was determined by measuring the Zeta potential. As shown in

Table 5, tests with CM-dextran [structure iv] showed that the negative charge could be fully

neutralized under appropriate reaction conditions.

Table 5

Sample	Zeta potential (mV)
Dextran T70[1]	-6.60
CM-Dextran[2]	-47.40
Ethanolamine neutralized CM-Dextran A[3]	-36.25
Ethanolamine neutralized CM-Dextran B[4]	-1.06

[1] Neutral.
10   [2] Degree of substitution ~ 50% determined by atomic absorption sodium analysis.
[3] EDC at 2 fold excess and ethanolamine at 10 fold excess of glucose units present on dextran.
[4] EDC at 10 fold excess and ethanolamine at 20 fold excess of glucose units present on dextran.

By tuning the amount of EDC and ethanolamine, two dextran-PVGLIG-methotrexate

conjugates with different negative charges were prepared. The kinetics of MMP-2 digestion

15   were also assessed as shown in Table 6.

Table 6

Sample	Zeta potential (mV)	k_{cat}/K_m ($M^{-1}.s^{-1}$)
Conjugate C1	-27.8	1.09×10^4
Conjugate C2	-12.2	1.22×10^5

The sensitivity constant of the less negative conjugate C2 was comparable to the value of

0.82×10^5 that was measured by Turk with the optimal oligopeptide Ile-Pro-Val-Ser-Leu-Arg-

Ser (Turk et al., *Nature Biotechnology* 19:661, 2001). Interestingly, none of the peptide motifs

5   of the MMP-2 recognition sequence found in the literature carry a negatively charged amino acid

(Turk et al., *Nature Biotechnology* 19:661, 2001; Netzel-Arnett et al., *Analytical Biochemistry*

195:86, 1991; Knight et al., *FEBS Letters* 296:263, 1992; Xia et al., *Bioch. Biophys. Acta*

1293:259, 1996). These reports are in agreement with the observation that the conjugate with the

least negative backbone charge demonstrates the fastest digestion with MMP-2.

10

Example 9 - *Peptidyl methotrexate release in the presence of MMP-2*

Dextran-PVGLIG-methotrexate and dextran-methotrexate conjugates from Example 8

were incubated with 14.7 nM active human MMP-2 in MMP-2 assay buffer at 37 C for 6 hours

and assayed for percentage cleavage using size exclusion chromatography. Dextran-PVGLIG-

15   methotrexate conjugate released more than 90% of methotrexate whereas dextran-methotrexate

did not show any significant release. The comparison confirms that the labile oligopeptide

segment promotes cleavage of the conjugate. Without the recognition segment, the conjugate

was stable even in the presence of a high concentration of MMP-2. The small molecular weight

cleavage product was collected from the digest of dextran-PVGLIG-methotrexate by size

exclusion chromatography and analyzed by mass spectroscopy. The molecular weight of 707.5

5   was consistent with the notion that methotrexate-Pro-Val-Gly (MTX-PVG) was the released

product after cleavage.

Example 10 - *Stability of dextran-oligopeptide-methotrexate conjugates in serum*

        As noted previously it is preferable that the conjugates remain stable in blood circulation

10   and that the drug stays attached to the polymer backbone before reaching the target tissue. When

tumor tissues express a high level of MMP-2, there will be an elevated enzyme level in the blood

stream (Kuyvenhoven et al., *Thrombosis and Haemostasis* 89:718, 2003). However, protease

activity is inhibited by serum proteins such as α-2-macroglobulin (Woessner and Nagase, *Matrix

Metalloproteinases and TIMPs*, Oxford University Press, 2000). The stability of dextran-

15   PVGLIG-methotrexate conjugates of Example 8 were therefore measured in both fetal bovine

serum and fetal bovine serum spiked with active human MMP-2 (at 10 nM final concentration).

After incubating the conjugates at 37 C in these conditions for 24 hours, no significant cleavage

was detected as assayed by size exclusion chromatography.

20   **Example 11** - *In vitro cytotoxicity of peptidyl methotrexate*

        As discussed in Example 7, the cytotoxicity of peptidyl methotrexate and free

methotrexate were found to be comparable when tested on three human tumor cell lines (see also Figures 11A-C).

Example 12 - *In vivo evaluation of anti-tumor efficacy of dextran-oligopeptide-methotrexate*

5　*conjugates*

The following example describes experiments that were performed to test the in vivo efficacy of the dextran-oligopeptide-methotrexate conjugates of Example 8. Six week old female SCID mice (obtained from Charles River Laboratory) were subcutaneously injected with 10^6 HT-1080 tumor cells (obtained from ATCC) at mid-dorsal. HT-1080 is a human

10　fibrosacroma cell line that is known to express high level of MMP-2. After 7-10 days, tumors of size 150-300 mm³ were established on each mouse. Free methotrexate, dextran-oligopeptide-methotrexate (with MMP-2 labile oligopeptide segment Pro-Val-Gly-Leu-Ile-Gly) or dextran-methotrexate were injected intraperitoneally on day 1, 8 and 15 after a tumor was first established. Weight and tumor size were monitored three times a week. Tumor size was

15　calculated as width² x length x 0.52. To ensure that the modified dextran carrier of the conjugates did not induce toxicity, a first control group of mice were injected with dextran that was charge neutralized with ethanolamine. As a second control, another group of mice were injected with phosphate buffered saline. Each group included three mice.

The dosage chosen for these experiments was based on the reported optimum dosage of

20　free methotrexate for treating tumor-bearing mice (Burger et al., *Int. J. Cancer* 92:718, 2001). Three weekly injections at 50 mg of methotrexate/kg of body weight was half the maximum

tolerated dosage. In another set of experiments the dosage was increased to three weekly

injections of 100 mg/kg, or to injections of 50 mg/kg twice per week. These higher dosages

resulted in serious toxic or lethal responses and body weight dropped more than 20% (data not

shown).

5   Figure 13 shows the tumor size progression after treatment, as an indication of treatment

efficacy. Free methotrexate suppressed tumor growth by 48% at the end of the study compared

to saline control. Dextran-methotrexate and dextran-oligopeptide-methotrexate conjugates were

both more effective in hampering the progression of tumor. At the end of study, average tumor

size in mice treated by either conjugate was suppressed by 92%.

10   Linking methotrexate to dextran increases the half-life of the small molecule drug due to

decreased renal elimination, rendering the benefit of passive targeting. As described above, the

leakiness of tumor blood vessels as compared to other normal tissue further increases the

targeting ratio. As an anti-metabolite, methotrexate must be released from the carrier to exert its

effect on cell growth by inhibiting DNA synthesis. The release can happen in two ways, namely

15   (1) by non-specific endocytosis, whereby conjugates are internalized by cells and methotrexate

can be released by either acid hydrolysis or lysosomal enzyme digestion; and (2) by MMP-2

cleavage of the oligopeptide segment in the extracellular space of the tumor tissue. The latter

route is only possible for conjugates with an MMP-2 labile oligopeptide segment between

methotrexate and the dextran carrier.

20   The body weight of the mice was also monitored as an indicator of toxic side effects (see

Figure 14). The polymeric carrier, modified dextran, showed no toxicity. At equivalent dosage,

methotrexate and dextran-oligopeptide-methotrexate caused similar percentage weight drops

(13% and 14% respectively compared to initial weight). The significantly better efficacy of

dextran-oligopeptide-methotrexate here demonstrates the advantage of targeting. The enhanced

anti-tumor effect of dextran-oligopeptide-methotrexate is presumably a result of altered

5   biodistribution of methotrexate, with more drug delivered to the targeted tumor tissue. Free

methotrexate is a drug of low molecular weight (MW = 454.45 Da) and has a short plasma half-

life. As a result, the total body clearance is high and only small amount of the injected dose

reaches the tumor tissue. The dextran-methotrexate and dextran-peptide-methotrexate

conjugates behave differently. Because the dextran carrier is larger than the renal excretion

10   limit, the plasma half-life is longer and this enables a larger fraction of the injected dose to reach

the tumor tissue. This advantage is termed passive targeting.

Dextran-methotrexate, albeit showing promising efficacy, was significantly more toxic

than dextran-oligopeptide-methotrexate, causing a 27% drop in body weight. In fact, the study

for this group was terminated at an earlier stage because of severe toxicity (Day 13, see Figures

15   13 and 14). It is speculated that the MMP-2 labile linker is responsible for the lower toxicity of

the dextran-oligopeptide-methotrexate. Indeed, in normal tissue without MMP-2, free

methotrexate from dextran-methotrexate conjugate is potentially released as a result of non-

specific endocytosis. In contrast, peptidyl-methotrexate is released from dextran-oligopeptide-

methotrexate conjugates. Because of the lower cytotoxicity of the peptidyl-methotrexate versus

20   the free drug, a lower toxic response is seen in normal tissues.

If the tumor associated enzyme MMP-2 mediates targeted drug release for the new

dextran-oligopeptide-methotrexate conjugate, one would expect a conjugate that is more

sensitive to MMP-2 in vitro to generate a more pronounced therapeutic effect. The in vivo

efficacy of the two dextran-oligopeptide-methotrexate conjugates of Table 6, Example 8 was

5   therefore compared as illustrated in Figure 15. Conjugate C2 that is more sensitive to MMP-2

was more effective in suppressing tumor growth. Tumor size was approximately the same as the

initially established size by the end of the study. In comparison, a tumor size increase of 40%

was observed when the mice were treated with the less sensitive conjugate C1. In addition

conjugate C2 was slightly less toxic than C1. Mice treated with conjugate C2 lost 8% body

10   weight while mice treated with C1 lost 13% by the end of study (data not shown).

Other Embodiments

The foregoing has been a description of certain non-limiting preferred embodiments of

the invention. Those of ordinary skill in the art will appreciate that various changes and

15   modifications to this description may be made without departing from the spirit or scope of the

present invention, as defined in the following Claims.

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